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ACCUMULATION OF FREE FATTY ACIDS FROM SEA WATER BY MARINE INVERTEBRATES

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Numerous investigators have reported uptake of dissolved free amino acids, glucose, and other small organic molecules from sea water by marine invertebrates (reviewed in Stephens, 1971). Other than the brief observations by Southward and Southward (1970) on uptake of palmitic and butyric acid by three species of Pogonophora, there are no published investigations on the ability of free-living marine invertebrates to take up fatty acids from sea water.

Dissolved fatty acids are available in the marine environment. Slowey, Jeffrey and Hood (1962) found fatty acids of 12–18 carbon number in ethyl acetate extracts of millipore filtered sea water taken from the Gulf of Mexico. Free fatty acids, long chain hydrocarbons, fatty acid esters, sterols, and many other substances, including phosphorus and nitrogen containing compounds which may have been phospholipids, were identified by Jeffrey, Pasby, Stevenson and Hood (1963). Total concentration of ethyl acetate/petroleum ether-extractable substances in these inshore Gulf samples was 4–14 mg/liter. Jeffrey (1966) hydrolyzed lipids and identified acids having less than 10 to 22 carbon atoms, with up to 6 unsaturated linkages in the longer acids. Total dissolved lipid concentrations ranged from 0.5 to 6.0 mg/l, the lower values being in the offshore Gulf samples. Williams (1965) found only 1–9 μg/l of dissolved fatty acids in samples from within and just outside of Puget Sound.

In view of the existence of fatty acids in the marine environment, it seemed desirable to determine if soft-bodied marine invertebrates other than pogonophorans could take up and assimilate free fatty acids. When preliminary experiments demonstrated the ability of several polychaetous annelids to accumulate C^{14} in the form of palmitic and oleic acid, it became of particular interest to determine whether a "saturable" uptake system was involved, in which the velocity is limited by the availability of a finite number of transport sites. Inhibitory interactions among fatty acids were investigated to cast light on the specificity of the uptake mechanism. Labelled animals were extracted and fractioned into the major biochemical classes by solvent partition and thin-layer chromatography in order to determine if the free fatty acids participate in energy metabolism and synthetic pathways.

Accumulation of a radioactive label is not adequate evidence that net accumulation of the compound occurs. When exchange diffusion or label exchange are operating, an organism may show initial accumulation of label even though the compound is leaking out faster than it is being taken up. The disappearance of

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unlabelled free fatty acid from the medium was monitored in order to check for this possibility.

Since uptake rate is a function of substrate concentration, once this function has been defined, data on the concentration of fatty acids in the natural environment of the organism can be used to make an estimate of the significance of the uptake process relative to the metabolic requirements of the organism. In view of the variability of fatty acid levels reported in the literature and the failure of these reports to specify how much of the fatty acid was in the free or combined form, it was deemed desirable to collect sea water samples from the habitat of the organism being studied and assay them for free fatty acids.

Materials and Methods

Stauronereis rudolphi (Della Chiaje) (Annelida, Polychaeta, Dorvilleidae) is especially plentiful during the summer and fall months among the mats of green algae and associated detritus that coat pilings and logs floating in Los Angeles Harbor. The animals burrow into the substrate and ingest it, but do not maintain permanent burrows (Reish, 1959). Specimens collected from the harbor were kept at 20° C in gallon jars of aerated and continuously filtered sea water and fed pellets of dried alfalfa ad lib. Worms were used within six weeks of collection.

Nainereis dendritica (Annelida, Polychaeta, Orbiniidae) abounds at and below the low tide line on exposed sandy beaches. Organisms were collected from under roots of *Phyllospadix* one mile south of Corona Del Mar and kept at 15° C in a large bucket of aerated sea water with several inches of sand in the bottom. Specimens were used within three weeks of collection.

Oleic, palmitic, stearic and linoleic acids labelled in the 1-carbon position with C^{14} were supplied by Amersham/Searle together with data attesting to at least 98% radiochemical purity. The claimed 99% purity of uniformly labelled palmitic acid from the same company was affirmed by thin-layer chromatography (TLC) on silica gel in pertoleum ether, ethyl ether, acetic acid: 70:30:2 (v/v/v). Caproic acid-1-C^{14} was obtained from Calbiochem and the acetic acid-1,2-C^{14} was supplied by Volk Radiochemical.

Accumulation of C^{14}-labelled free fatty acids

Glass experimental vessels were coated with "Siliclad" (Clay-Adams, Inc.) to minimize fatty acids sticking to the glass. Teflon beakers were also used in some experiments.

Artificial sea water (M. B. L. S. W.) made up in distilled water from reagent grade salts according to the standard Marine Biological Laboratory, Woods Hole, formula (Cavanaugh, 1964) was millipore filtered twice just before use. Animals were allowed to remain at least 24 hours in clean M. B. L. S. W. to empty the gut before transfer to the experimental beakers. Small worms such as *Stauronereis* were incubated in groups of 1 to 2 dozen so that each beaker contained about 300 mg of animal material. Enough radioactive fatty acid was introduced into each 50 ml beaker of M. B. L. S. W. to give an activity of 20 μc/l. Carrier for the radioactive stock was 0.1 ml of 85% ethanol. Unlabelled fatty acid (supplied by Calbiochem) was added to bring the solution up to the desired concentration.

After incubation, rinsing and weighing, the worms were dropped into scintillation vials containing 10 ml of scintillation fluid (6 grams PPO/l toluene, 2 parts added to 1 part Triton-X detergent) and left 2 days before counting. If quenching was too great, the extract was diluted 10 × before counting. This method extracted 97% of the radioactivity in the worms. Larger invertebrates were digested with formic acid. The vials were counted in a Beckman CPM-100 liquid scintillation spectrometer. Efficiency of C^{14} counting of experimental samples was approximately 80%.

Assimilation of palmitic acid-U-C^{14}

Six specimens of *Naineris dendritica*, weighting an average of 110 mg each, were preincubated 24 hours in M. B. L. S. W. containing 200 mg streptomycin sulfate per liter. M. B. L. S. W. containing 40 μcuries/l palmitic acid-U-C^{14} (supplied by New England Nuclear) was used as the incubation medium. Homogenization and extraction were according to the standard procedure of Roberts, Abelson, Cowie, Bolton and Britten (1963), which separates the tissue into fractions soluble in cold 5% trichloroacetic acid (TCA), 85% ethanol, ether, hot TCA, and a residual protein precipitate. The ether-soluble fraction was chromatographed along with suitable standards on Merck 250 μ silica gel "G" plates (Brinkmann Instruments, Inc.) developed in chloroform, methanol, water: 65 : 25 : 4 (v/v/v). The radioactive spots were located by autoradiography, then scraped into scintillation vials and counted.

Loss of unlabelled oleic acid from the medium

Unless otherwise noted, all organic solvents used in this and the following experiments were Mallinckrodt "nanograde" (glass distilled, nonvolatile matter $< 5 \times 10^{-4}$%). All glassware was cleaned in chromic acid, coated with Siliclad, rinsed in glass redistilled water and then with hexane. The M. B. L. S. W. was made up from reagent grade salts, glass redistilled water, and was millipore filtered three times before use.

50 specimens of *Stauronereis rudolphi* were cleaned by preincubating them for 24 hours in M. B. L. S. W. to which streptomycin sulfate was added to make a concentration of 200 mg/l. The worms were then rinsed and placed into one of two teflon beakers, each of which contained 50 ml of M. B. L. S. W. in which was dissolved 2.5 μmoles oleic acid per liter. Duplicate 10 ml samples were taken from the control beaker at the beginning of the experiment and from both beakers at the end of one hour. The water samples were acidified with 2 N HCl and extracted 3 times with 2 ml portions of hexane. The extracts were pooled into conical centrifuge tubes, evaporated to dryness under N_2, redissolved in 0.1 ml benzene and quantitatively spotted onto Analtech 250 μ silica gel "G" thin-layer plates which had been divided into 7 mm lanes (method of Downing, 1968). The plates were developed in hexane, ether, acetic acid: 60 : 40 : 2 (v/v/v), sprayed with 50% H_2SO_4 (v/v) and heated 20 minutes in a 180° C oven to char the lipid spots. The free fatty acid spots were scanned with a Photovolt Model 520A densitometer using a 396 mμ filter. The product of the peak width at ½ height times the height was a linear function of the weight of oleic acid standard (TLC neutral lipid standard, Sigma Chemical Co.) for values above 2 μgrams.

Analysis of dissolved lipids

Sea water sample "A" was collected in a glass bottle on September 28, 1970, 1 foot below the surface near Berth #158 of Los Angeles Harbor. The 1500 ml sample was put on ice and immediately brought back to the laboratory and filtered with suction through multiple sheets of Whatman #1 filter paper and then through Whatman GF/A glass fiber paper. The filtrate was brought to about pH 2 with 2 N HCl and extracted 3 times by shaking with 150 ml portions of chloroform. The extracts were filtered through chloroform-extracted Whatman 1-PS phase separating paper to remove droplets of water. Two grams of chloroform-extracted Sephadex G-25/40 were added to remove water, salts and non-lipid contaminants according to the method of Williams and Merrilees (1970). After evaporation of the solvent under vacuum, the powder was quantitatively washed onto a sintered glass filter with a little chloroform and the lipid material was eluted with chloroform. The filtrate was evaporated to dryness under a stream of N_2, dissolved in 0.5 ml benzene and stored under N_2 at −20° C. A solvent blank was prepared by treating 500 ml of chloroform in the manner described above for the extract.

Sample "B" was collected on October 1, 1970, at the same location by drawing water from under the algal and mussel mat on floating pilings using a large syringe. The 2 liter sample was treated as the first sample, except that particulates were removed by centrifugation at 0° C in Siliclad coated Nalgene buckets for 25 minutes at 11,600 G (*cf.* Rudolfs and Balmat, 1952).

The chloroform extraction removed more than 99% of palmitic acid-C^{14} dissolved in filtered M.B.L.S.W. Glass fiber filtration removes about half of organic acids dissolved in sea water (Quinn and Meyers, 1971). Recovery of oleic acid-C^{14} added to sample "B" sea water before centrifugation was also about 50%. Virtually no activity was retained by the Sephadex powder after elution with chloroform.

Measured aliquots of the sea water extracts and solvent blank were chromatographed on Analtech thin-layer plates, charred and scanned as above. Peaks were identified and quantified by comparison with Sigma quantitative neutral lipid standards.

The rest of the samples were saponified by heating at 60–80° C for 30 minutes with 1 ml 10% KOH in 50% methanol and the non-saponifiable substances removed by hexane extraction. The mixture was then acidified, 2 ml of water were added, and the fatty acids extracted with hexane. Esterification was carried out by the procedure of Morrison and Smith (1964). After 0.5 ml of benzene and 2 ml of 14% BF_3 in methanol (Applied Sciences Laboratories) were added to the fatty acids, the mixture was heated at 80° C for 15 minutes. 2 ml of water were added to the cooled reaction mixture and the methyl esters extracted 3 times with hexane.

Gas-liquid chromatography (GLC) of the fatty acid methyl esters was done using a Barber-Colman Model 5000 instrument employing a single argon ionization detector. The columns, glass U-tubes 6 feet long and 3.5 mm in inside diameter, were packed with either 10% Apiezon-L on 60/80 mesh Gas Chrom Q or 10% ethylene succinate methylsilicone polymer (EGSS-X) on 100/120 mesh Gas Chrom P (Applied Sciences Laboratories, Inc.). The Apiezon column was

operated on a temperature program starting at 175° C and rising to 270° C at the rate of 2° C/minute. Argon gas flow was 60 ml/minute and the injector port and detector were heated to 280° C and 300° C, respectively. The EGSS-X column was operated isothermally at 180° C with carrier gas flowing at 50 ml/minute and injector and detector at 220 and 235° C, respectively. Under these conditions the logarithm of the retention distance is a linear function of the carbon number (James, 1960). Chromatographing the same sample on both columns facilitates identification of the peaks, as the unsaturated esters run ahead of their saturated analogues on the non-polar Apiezon column, and behind the

TABLE I

Species surveyed for ability to take up dissolved free fatty acids. Unless otherwise noted, the organisms were incubated for 1 hour in M.B.L.S.W. containing a C^{14}-labelled fatty at the concentration specified

Species	Fatty acid	Conc. (μmoles/l)	cpm/g tissue / cpm/g medium	Per cent of medium activity lost	Per cent recovery in organism
Nainereis dendritica	palmitic	0.10	24.6	34	66
	oleic	0.60	56.4	45	66
Stauronereis rudolphi	oleic	0.35	27.4	34	75
Glycera dibranchiata	palmitic	0.14	1.8	18	35
Podarke pugettensis	palmitic	0.08	16.2	28	35
Lumbrineris spp.	palmitic	0.12	4.6	9	42
Cirriformia spirabrancha	palmitic	0.12	4.0	23	49
*Nereis limnicola**	oleic	0.68	6.6	4	86
*Tubifex tubifex***	oleic	0.69	10.6	6	101
	caproic	0.50	12.7	12	95
Urechis caupo	oleic	0.34	3.1	52	92
Amphipholis pugetana	palmitic	0.09	4.4	18	55
Strongylocentrotus purpuratus	palmitic	0.18	3.6	18	45

* Incubated in 0.4% NaCl.
** Incubated in distilled water for ½ hour.

saturated esters on the polar EGSS-X column. The relative weight of each methyl ester was calculated by multiplying the peak height times the retention distance (Brandt and Lands, 1968) on the EGSS-X column and by comparison with K-101 and K-108 FAME standard mixtures supplied by Applied Sciences Laboratories.

RESULTS

Survey of fatty acid uptake

A number of species of marine worms, the fresh water oligochaete *Tubifex tubifex*, the echiuroid worm *Urechis caupo*, and 2 echinoderms were tested for ability to take up one or more C^{14}-labelled fatty acids. Most were able to concentrate the label at least severalfold over the medium activity (Table I). Radioactivity accumulated in the organism as it was lost from the medium. About 10% of the radioactivity in artificial sea water medium adhered to the surface of the glassware in control beakers containing no organisms. The rest of the radioactivity could be accounted for in the rinse water and mucus thrown off by some of the organisms.

In the first experiments 200 mg of streptomycin sulfate or a mixture of 200 mg streptomycin, 50 mg chloramphenicol, and 500,000 units of penicillin were added to each liter of medium to retard microbial activity. This practice was discontinued when it was found to make no difference in the rate of C^{14} uptake.

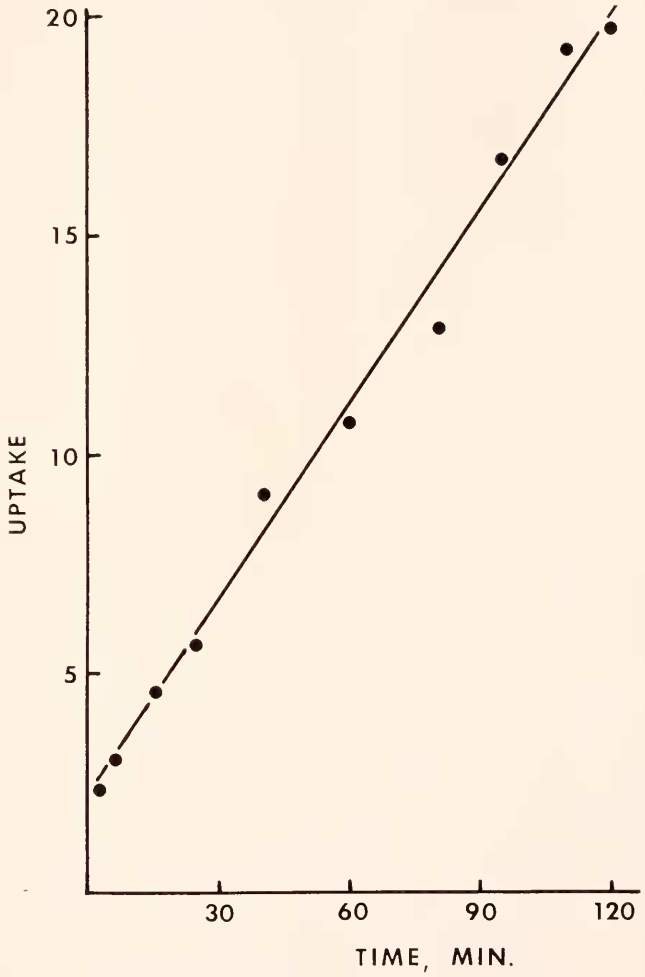

FIGURE 1. Uptake of linoleic acid-1-C^{14} from a 2.0 μM solution—activity per fresh weight of worm tissue (arbitrary units) as a function of incubation time. Each point represents a group of approximately 20 worms. Linear regression lines in this and the following figures were fitted by the method of least squares.

The uptake of C^{14} by live *Nainereis dendritica* exposed to 0.1 μmoles/l of palmitic acid-U-C^{14} was compared to that of individuals that had been killed with KCN. Live specimens were able to concentrate the label by a factor of 25 over the medium activity; dead controls were unable to concentrate the label more than 0.8 times.

The marine worm *Stauronereis rudolphi* was chosen for detailed kinetic studies. When uptake velocities in M. B. L. S. W. were compared to those measured in filtered sea water from L. A. Harbor, there was no difference in the velocity of palmitic acid uptake, but uptake of oleic and caproic acid from sea water was 25% and 50%, respectively, lower than from M. B. L. S. W.

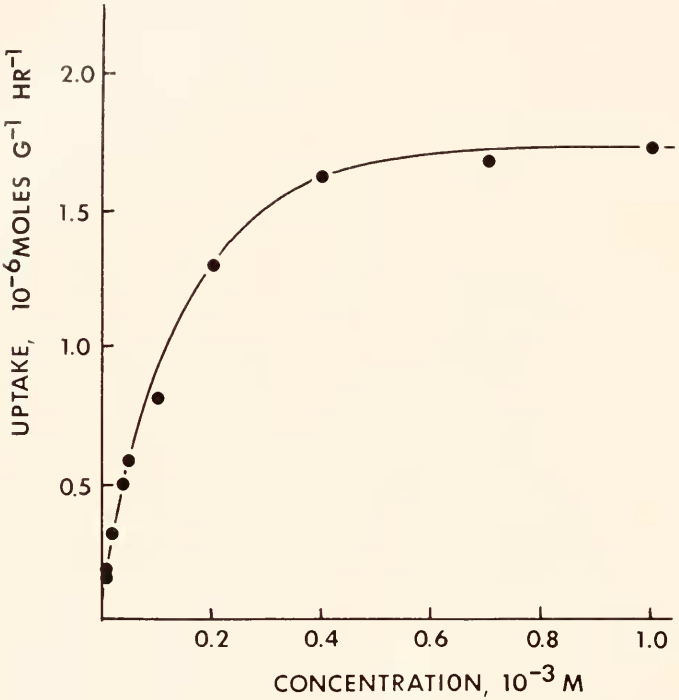

FIGURE 2. Uptake velocity of acetic acid-C^{14} as a function of substrate concentration.

Uptake as a function of body weight

Weight specific uptake of C^{14}-labelled oleic acid by *Stauronereis* decreased with increasing body weight such that the slope of a double logarithmic plot of uptake as a function of weight is 0.80. However, uptake rates in the following experiments were adjusted for variation in worm size simply by dividing the total tissue radioactivity by the fresh weight. The error inherent in this approximation was minimized by using worms of approximately the same size within any given series of experiments.

Uptake as a function of time

Uptake by *Stauronereis* of palmitic, oleic, linoleic, caproic and acetic acid proceeded linearly with time for the 2–3 hour incubation period. In the case of the first 3 acids, there was often an abrupt initial spurt of uptake in the first few minutes (see Fig. 1 for a particularly striking example). In the following

TABLE II

Michaelis-Menten constants for uptake of fatty acids by Stauronereis rudolphi *± 95% confidence intervals. N is the number of groups of worms used for the determination*

Fatty acid	N	K_s (10^{-6} moles/liter)	V_{max} (10^{-9} moles/g hour)
palmitic	8	1.09 ± 0.49	20.5 ± 5.4
palmitic	13	0.98 ± 0.38	13.0 ± 3.2
oleic	50	1.26 ± 0.36	41.3 ± 6.5
linoleic	31	18.4	621
caproic	17	12.5 ± 3.6	179 ± 39
acetic	10	102 ± 15	1900 ± 170

kinetic studies the initial spurt was ignored and uptake velocities were calculated after at least 1 hour of incubation to minimize the error.

Uptake as a function of substrate concentration

The graph of uptake velocity as related to ambient concentration of acetic acid (Fig. 2) reveals typical saturation kinetics which can be described by the Michaelis-Menten equation, $v = V_{max} \cdot S/(K_s + S)$, where v is the velocity, S is the substrate concentration, V_{max} equals the maximum velocity and K_s is numerically equivalent to the substrate concentration at which the velocity is one-half the V_{max}.

Uptake of the other fatty acids listed in Table II also follows saturation kinetics except for important deviations found at the higher concentrations of palmitic and oleic acids (Figs. 3 and 4A). When palmitic or oleic acid were introduced into

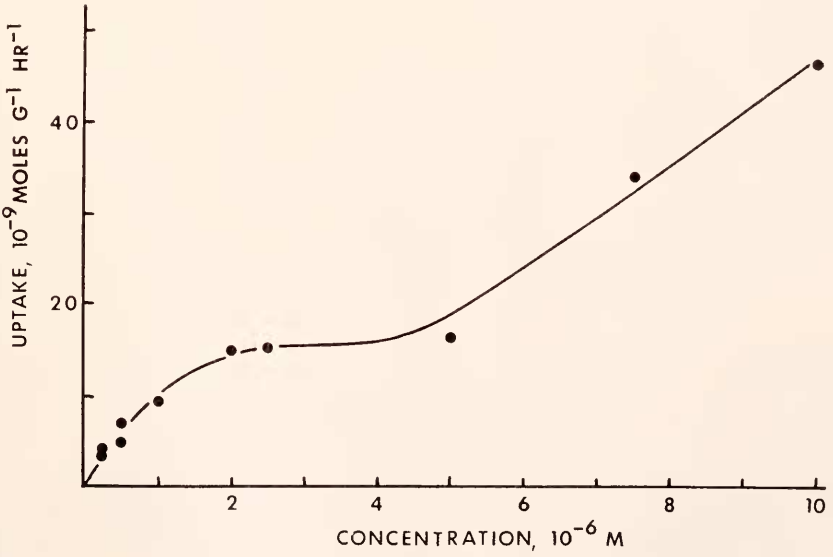

FIGURE 3. Uptake velocity of palmitic acid-1-C^{14} as a function of substrate concentration.

M. B. L. S. W., the solution remained clear up to a concentration of about 5 μmoles/l. Above this concentration a slight opalescence could be detected and further addition of fatty acid resulted in turbidity, which was associated with the anomalously high uptake velocities. Only data from concentrations of 5 μmoles/l

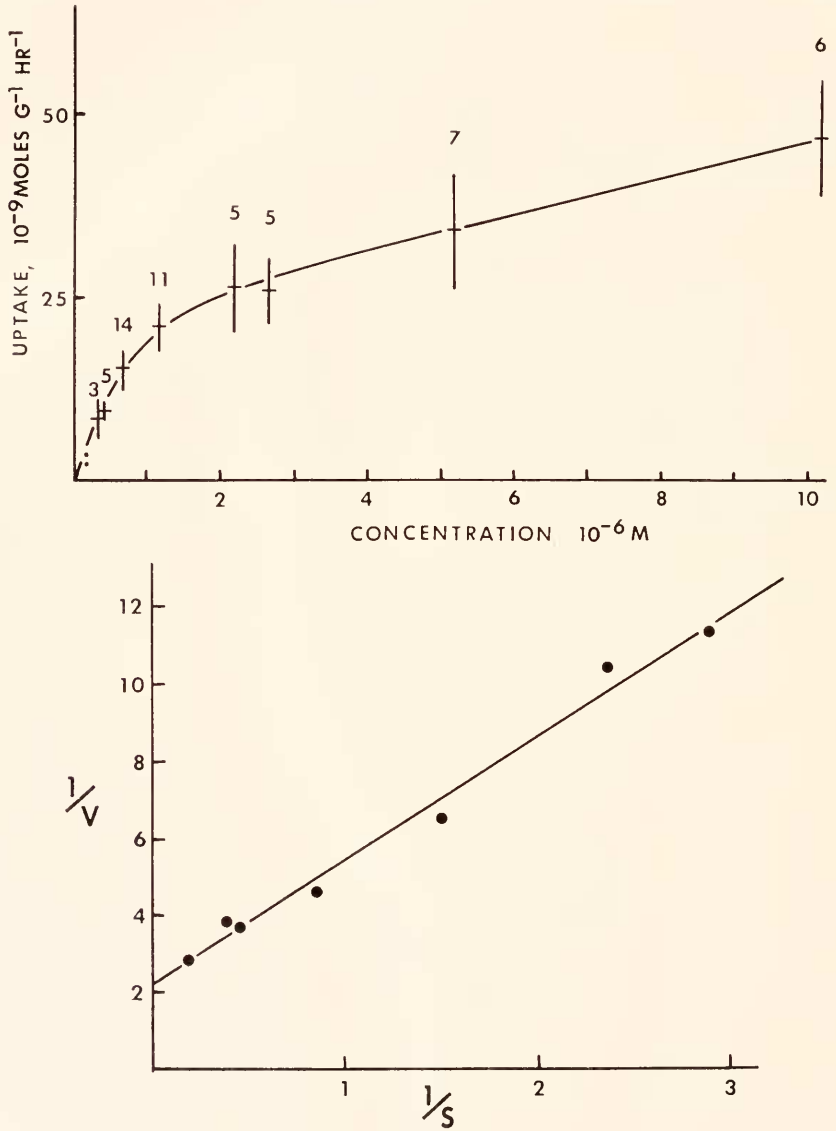

FIGURE 4. Uptake of oleic acid-1-C^{14}; (A.) velocity as related to substrate concentration. The vertical lines delineate the 95% confidence intervals and the numerals are the number of groups of worms used in each determination; (B.) Lineweaver-Burk transformation of the same data (where $V = 10^{-7}$ moles/g/hr and $S = 10^{-6}$ M), omitting the point at $S = 10^{-5}$ M.

and below were used in calculating kinetic parameters of palmitic and oleic acid. The turbidity problem with linoleic and caproic acid was much less severe. Concentrations up to 20 μmoles/l of linoleic acid and 30 μmoles/l of caproic acid could be used without difficulty.

When the reciprocal of the uptake velocity is plotted against the reciprocal of the substrate concentration (Lineweaver-Burk plot), a straight line results in which the y intercept equals $1/V_{max}$ and the slope equals K_s/V_{max}. The Lineweaver-Burk plot of oleic acid uptake is presented here (Fig. 4B) because kinetic data have traditionally been visualized in this manner. However, this method has the disadvantage of disproportionately emphasizing the data at lower substrate concentrations and giving less reliable Michaelis-Menten constants than the Hofstee (v *versus* v/S) or the Woolf (S/v *versus* S) plots of the Michaelis-Menten equation (Dowd and Riggs, 1965). The constants presented in Tables II and III were derived from linear regression lines calculated by the method of least squares for data plotted by one of the last two methods.

TABLE III

Uptake of oleic acid-1-C¹⁴ inhibited by various fatty acids. Michaelis-Menten constants ± 95% confidence intervals

Inhibitor	N	I (10^{-6} moles/l)	K_s (10^{-6} moles/l)	V_{max} (10^{-9} moles/g/hr)
none	50	—	1.26 ± 0.36	41.3 ± 6.5
linoleic	7	20	4.04 ± 2.02	43.8 ± 16.9
palmitic	16	10	1.89 ± 0.80	44.8 ± 10.7
caproic	20	20	2.73 ± 1.20	50.9 ± 15.1

Data were not adequate to compute Michaelis-Menten constants for stearic acid uptake. Average velocity of 8 groups of worms exposed to 0.48 μmoles stearic acid/l was 0.70×10^{-9} moles/g/hour, about $\frac{1}{5}$ the velocity of palmitic acid uptake at that concentration.

The V_{max} of oleic acid uptake in the presence of constant amounts of linoleic, palmitic or caproic acid was not significantly affected, but the apparent K_s increased (Table III). However, this was significant at the 95% confidence level only for linoleic acid. Oleic acid uptake was not inhibited by a concentration of acetic acid 300 times greater.

Assimilation of palmitic acid-U-C¹⁴

After 1 hour incubation 64% of the radioactivity remained in the ether-soluble fraction, 33% appeared in the cold TCA-soluble fraction and 3% in the remaining hot TCA-soluble, alcohol-soluble and protein fractions. The same percentages in the control were 79%, 20%, and 0.2%, respectively. TLC of the ether-soluble fraction revealed that only 28% of the radioactivity remained in the free fatty acid fraction, whereas 98% of the control's radioactivity remained in this fraction (the palmitic acid-C¹⁴ had been added to the control animals just prior to homogenization in cold 5% TCA). The rest of the radioactivity was incorporated by neutral lipids (48%) and phospholipids (23%).

After the 1 hour incubation, 2 of the worms were rinsed and sealed into flasks containing 20 ml of M. B. L. S. W. At the end of 1 hour they were removed, dropped into scintillation fluid, and the C^{14} activity measured in the liquid scintillation counter. Radioactivity leaked into the medium was measured both immediately and after the medium had been acidified and allowed to stand for 24 hours. Leakage of activity from the worms was only 4.0% and 5.1% of the uptake in 2 trials. 44% and 56% of the activity in the medium disappeared after acidfication of the samples, and was thus considered to be in the form of $C^{14}O_2$.

Loss of unlabeled oleic acid from the medium

The drop in medium concentration of oleic acid in the flask containing *Stauronereis* exceeded that of the control ($P < 0.05$) by 10.4 and 11.0 μgrams oleic acid per 10 ml sample in two separate experiments.

TABLE IV

Quantitative TLC of dissolved sea water lipids. Results are expressed as μgrams carbon per liter sea water ± 1 standard deviation. The values represent the mean of 4 determinations solvent blank

Rf	Lipid class	μgrams carbon per liter	
		Extract "A"	Extract "B"
0.08	monoglycerides	14.1 ± 1.2	5.8 ± 1.3
0.13	?	8.3 ± 0.5	trace
0.22	sterols	6.0 ± 0.8	5.7 ± 0.8
0.27	diglycerides	—	5.0 ± 1.0
0.31	?	12.9 ± 3.2	20.0 ± 2.7
0.35	free fatty acids	63.7 ± 4.7	51.4 ± 3.6
0.47	?	—	trace
0.52	triglycerides	5.2 ± 0.1	6.0 ± 2.0
0.85	sterol esters, hydrocarbons	—	38.0 ± 1.6
total		110	132

Analysis of dissolved lipids

The neutral lipids extracted from L. A. Harbor sea water are presented in Table IV. In addition to the substances listed in the table, there was a small quantity of material remaining at the origin that migrated in a chloroform, methanol, water: 65 : 25 : 4 solvent system. It included pigments and nitrogen-containing compounds that appeared to be phospholipids, but there was not enough sample left to make positive identifications. Note that about 50% of the neutral lipid material was in the free fatty acid fraction. Assuming an average carbon number of 16, the 58 μgrams of dissolved free fatty acid carbon per liter is equivalent to 0.30 μmoles/l. The solvent blank showed small but measurable amounts of compounds having Rf's that corresponded to free fatty acids, triglycerides, sterol esters and hydrocarbons, which were subtracted from the values measured in the sea water extract before presentation in the table.

Gas chromatography revealed a number of fatty acid methyl esters (Fig. 5 and Table V). The solvent blank contained mostly methyl esters of palmitic,

stearic and oleic acid, but the total quantity was only about 8% of that in the extract. The fatty acid compositions of sea water extracts "A" and "B" were so similar that the differences were within the range of experimental error and the two extracts were pooled for the analysis reported.

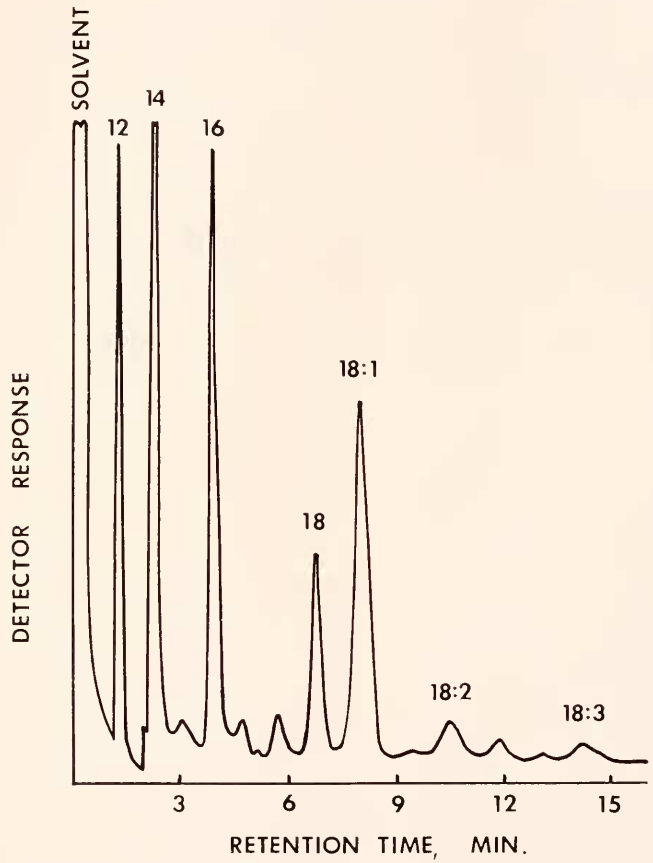

FIGURE 5. Gas-liquid chromatograph on the EGSS-X column of fatty acid methyl esters prepared from sea water extracts "A" and "B." The sudden shift in the tracing between the 12- and 14-carbon peaks represents a 3 times increase in detector sensitivity.

DISCUSSION

These studies demonstrate accumulation of dissolved free fatty acids by free-living marine invertebrates. Evidence comes from experiments on (1) the time course of uptake, (2) comparison of uptake rates of dead and live animals, (3) assimilation of labelled fatty acids, (4) net loss of unlabeled fatty acids from the incubation medium.

Uptake of C^{14}-labeled fatty acids proceeds linearly with time for at least 2 to 3 hours. An initial spurt of rapid uptake of long chain fatty acids is probably due to adsorption on the surface of the animals. Vavrečka, Poledne and

Petrásek (1966) found rapid initial uptake of palmitic acid-1-C^{14} into liver slices, which they attributed to surface adsorption. However, the failure of dead animals to accumulate significant amounts of label implies that the long term uptake process is due to some property of the living organism.

Anderson and Stephens (1969) showed that epiflora was responsible for apparent uptake of glycine by marine crustaceans. The same antibiotic mixtures that were effective in eliminating apparent uptake of glycine by crustaceans fail to inhibit fatty acid uptake by marine worms. Therefore it is unlikely that the fatty acid uptake could be attributed to the action of surface bacteria.

TABLE V

GLC on the EGSS-X column of methyl esters of dissolved fatty acids. Per cent composition is by weight. Retention times were measured from the air peak. The chromatogram was continued for 52 minutes, but no peaks appeared after those listed

Carbon number	Retention time minutes	Per cent composition
12	0.89	18.2
14	1.57	15.6
15	2.12	0.5
16	2.75	18.3
16:1	3.32	1.4
17	3.64	0.4
16:2	4.07	2.0
18	4.83	11.2
18:1	5.72	22.8
18:2	7.47	3.3
20	8.47	2.3
?	9.31	0.5
18:3	10.24	2.2
?	11.94	1.3

Analysis of worms exposed to C^{14}-labelled palmitic acid shows that the compound rapidly enters energy and biosynthetic pathways. Pocock, Marsden and Hamilton (1971) found evidence that free fatty acids function as a basic energy substrate in *Nereis virens* and probably have a very high turnover rate, as the animals contained only traces of free fatty acids. Leakage of isotopic label from *Nainereis* back into the medium was slight, but this gives little indication of the true rate of fatty acid leakage, as the identity and specific activity of the leaked compounds are not known.

Extraction and assay of oleic acid containing medium demonstrates that there is net loss of higher free fatty acids from the medium in the presence of *Stauronereis*. Uptake velocities measured by two independent techniques are roughly in agreement. Unlabelled oleic acid disappeared from the medium at the rate of 37.9×10^{-9} moles/g/hour. The velocity calculated from the rate of oleic acid-C^{14} accumulation at the same concentration is 26×10^{-9} moles/g/hour.

Uptake appears to occur across the body wall rather than the gut. A dozen specimens of *Stauronereis rudolphi* weighing a total of 300 mg can easily remove a third or more of the radioactivity in 50 ml of M. B. L. S. W. in an hour.

This would imply a rate of water movement through the gut for which there is no evidence. They were never observed to pump water through the gut. The dark gut contents, which can be seen against a light, take a matter of hours or days to pass out after the worms have been put into clean water. The slope of the double logarithmic plot of uptake as a function of weight, which is less than one, indicates that we may be dealing with a property related to the surface of the organism (*cf.* Reish and Stephens, 1969).

The uptake mechanism of oleic and palmitic acid appears to have two components. However, attempts to apply a "diffusion" correction to the data resulted in distortions of the data at lower concentrations, which suggested that the second component operates only at the higher concentrations. The second component correlates with the appearance of turbidity in the medium, which makes more likely an interpretation relating the anomalously high uptake velocities with a physical change of the fatty acid solution rather than attributing them to a property of the membrane. Palmitic acid, which has the most marked increase of uptake rate, is the least soluble fatty acid used in the kinetic experiments. The phenomenon is absent in the case of caproic and acetic acids, which are both very soluble. Chappell, Arme, and Read (1969) found evidence that palmitate uptake in the tapeworm *Hymenolepis diminuta* is enhanced by the presence of molecular aggregates. Bailey and Fairbairn (1968) noted more rapid uptake of oleic acid by *Hymenolepis* from micellar solution. Hoffman (1970) compared uptake of oleic acid-C^{14} by everted sacs of rat jejunum above and below the critical micelle concentrations of 2 detergents, one a bile salt and the other a high molecular weight non-ionic detergent. Uptake was greater from micellar media of both detergents, indicating an effect of micellar phase *per se* in promoting uptake, possibly by serving as a pool to maintain the concentration gradient of fatty acid at the transport surface. He also demonstrated that uptake is by single molecules rather than as intact micelles. A similar mechanism with respect to palmitic and oleic acid uptake in *Stauronereis* could account for the present data.

Southward and Southward (1970) also encountered anomalously high uptake of palmitic acid by the pogonophore *Siboglinum ekmani* at the same concentrations that I did with *Stauronereis*. When they eliminated the anomalous points they found a double logarithmic relationship of uptake velocity and palmitic acid concentration from 5×10^{-9} M to 6×10^{-6} M. The authors suggested pinocytosis as a possible uptake mechanism. They measured uptake velocities of 10^{-8} moles/g/hour from a 10^{-6} M palmitic acid solution, which falls within the range of velocities that I measured for palmitic acid uptake by *Stauronereis* at that concentration. Sodium butyrate was taken up much more slowly by *Siboglinum atlanticum* ($V_{max} = 2.7 \times 10^{-9}$ moles/g/hour) and had a much lower K_s (3×10^{-9} M) than uptake of short chain acids by *Stauronereis*, yet they found saturation of the uptake mechanism at higher concentrations.

V_{max}'s vary and tend to decline as the condition of the worm cultures deteriorates, but K_s's remain relatively constant. Two separate determinations of the Michaelis-Menten constants for uptake of palmitic acid are given in Table II as a typical illustration of the phenomenon. This is compatible with a model that attributes uptake to a mediated transport system having a finite number of active sites on the surface of the organism. The affinity of a given site for the

substrate, which is expressed as the K_s, is a constant property of the transport molecule, whereas the V_{max} varies with the number of functioning sites, which is inconstant.

The increase of apparent K_s for oleic acid uptake in the presence of linoleic, palmitic, or caproic acid while the V_{max} remains unchanged shows that the inhibition is competitive. This implies a common transport pathway for these fatty acids. The failure of acetic acid to inhibit oleic uptake suggests a separate site for uptake of acetate.

The dissolved lipid composition of the sea water samples is unusual in that such a large percentage consists of free fatty acids. Lela Jeffrey of Texas A&M University (personal communication) considered that only about 1% of the lipid substances passing a 0.45 μ filter are free fatty acids. Garrett (1967) reported 1–25% free fatty acids in lipids found in surface slicks. The recovery of free acids may depend to a large degree on the method used in handling the samples. The silicone treatment of all glassware used in handling my sea water samples may have increased recoveries of the free acids, as fatty acids have a tendency to stick to untreated glass surfaces (Green, 1969). Quinn and Meyers (1971) reported that recovery of heptadecanoic acid from sea water after passage through Whatman 54, Whatman GF/C glass fiber or Millipore HA membrane filters was 98.5, 51.9 and 14.2 per cent, respectively. Millipore membrane filters have been used by nearly all workers reporting dissolved lipids. Jeffrey used glass fiber paper only in the work reported in her 1970 paper, and Garrett (1967, page 223) did not filter his samples "to avoid losses of surface-active material due to selective adsorption onto the filter." Max Blumer of the Woods Hole Oceanographic Institute (1970) reported 19.7 μg/l total fatty acids in Buzzards Bay, Mass. sea water that he filtered through Whatman 54 paper. He estimated from IR spectra (personal communication) that roughly one-half was in the free acid form.

A puzzling feature of the fatty acid spectrum is the absence of the higher polyunsaturated fatty acids that are characteristic of marine lipids. Jeffrey (1966) reported dissolved fatty acids having up to 22 carbon atoms and as many as 6 double bonds in the longer acids. It is possible that much of the dissolved lipid material in the upper reaches of the harbor comes from leaching of sediments, fresh water runoff and organic pollution. Palmitic acid is almost always the most abundant fatty acid in marine sediments, which are also characterized by the virtual absence of polyunsaturates (Parker, 1967). Peterson (1967) found that the 14-, 16-, and 18-carbon acids dominated the sedimentary fatty acids and 16:1 and 18:1 were the dominant unsaturated acids. The dissolved fatty acids in L. A. Harbor exhibit a similar composition (Table V).

Hunter and Heukelekian (1965) found that the soluble organic matter in domestic sewage was mostly ether soluble, of which 56% was lower organic acids. Sewage pollution could explain the presence of C_{12} and C_{14} fatty acids in amounts approaching palmitic acid. My methods of extraction and analysis would not detect organic acids of less than 12 carbon atoms, but their presence might explain the 50% inhibition by harbor sea water of caproic acid-C^{14} uptake.

Although V_{max}'s of fatty acid uptake by *Stauroneis* are very low, K_s's are also low, allowing transport to take place efficiently from substrate concentrations in the submicromolar range. The V_{max} and K_s of palmitic acid uptake are about

3 orders of magnitude lower than reported for the intestinal parasite *Hymenolepis diminuta* by Chappell, Arme, and Read (1969). This suggests an adaptation to the low environmental concentrations encountered by the free-living worm. As a nutritional source, however, the process makes only a small contribution to the metabolic needs of the organism. Assuming a QO_2 of 0.05 ml O_2/gram/hour, the percentage of oxygen uptake that could be supported by accumulation of free fatty acids by *Stauronereis* at the concentrations measured in L. A. Harbor sea water is: palmitic acid, 2.0% ; oleic acid, 4.5% ; linoleic acid, 0.8%. If one assumes that the other fatty acids are taken up in proportionate amounts, uptake of total long-chain fatty acids could support 16% of the worm's oxidative metabolism. It should be noted, however, that the actual nutritional needs of the organism are likely to be considerably greater than that necessary to support the oxygen consumption in order to allow for growth, gamete production and anaerobic metabolism (see Stephens, 1963). The contribution would be greater in areas having higher fatty acid concentrations. To my knowledge, there have been no other published quantitative analyses of dissolved free fatty acids in inshore waters or recent marine sediments.

Several possibilities other than a reduced carbon source suggest themselves. Uptake could be a source of specific fatty acids, phospholipids, or a route for entry of pheromones with fatty acid moities. It is tempting to speculate about possible uptake of synthetic soaps, detergents, pesticides and other man-made lipid substances, although these substances have not yet been investigated.

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Summary

1. It has been established that representative marine annelids can accumulate and metabolize dissolved free fatty acids.

2. Net uptake of fatty acid from dilute solution by the polychaete *Stauronereis rudolphi* was confirmed by the loss of total higher fatty acids from the medium.

3. Uptake takes place across the body wall by a mediated transport system having kinetic properties analagous to those of enzyme-catalyzed reactions, including saturation at high substrate concentrations and competitive inhibition.

4. At concentrations above 5 μmoles/l, palmitic and oleic acids are taken up at anomalously high rates probably due to the formation of micelles or molecular aggregates.

5. Dissolved free fatty acids occurring in Los Angeles Harbor sea water were extracted and analyzed by thin-layer and gas chromatography.

6. Although the K_s's of higher fatty acid transport are low, allowing efficient uptake from low concentrations, the levels of free fatty acids found in the natural

environment are probably too low for uptake to support more than a few per cent of the organisms's metabolism.

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